Summary of Conclusions.

1. The *Spirillum Theileri* is naturally transmitted by the progeny of *Rhipicephalus decoloratus* which have developed on cattle suffering from or recovered from Spirillum infection.

2. It is possible to produce spirillosis-susceptible cattle and sheep by the injection of blood from sick or immune animals. The proof that the blood of immune sheep is infective is yet wanting.

3. The pathogenic effect of Spirillum is a slight anæmia accompanied by fever. In none of my cases did a fatal result occur.

An Experimental Enquiry into the Nature of the Substance in Serum which influences Phagocytosis.

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Metchnikoff, and his school, in the face of much opposition, lasting many years, have offered convincing proofs of the importance of phagocytosis in the protection of the animal body against bacterial invasion. The main theses of the Metchnikovian theory are now almost universally accepted, but the exact mechanism of the processes involved is even now the subject of keen controversy. If a highly virulent organism is injected into a susceptible animal, the leucocytes appear to be repelled, and to be unable to deal with the microbe, which multiplies and causes the death of the animal. If, however, the suitable immune serum is injected into the animal before inoculation, the phagocytes attack and devour the invading micro-organisms.

Much discussion has centred round the interpretation of such experiments. The early work of Nuttall and others on the bactericidal action of normal serum, and Pfeiffer's demonstration of the bacteriolysis of cholera and typhoid bacilli by immune sera in the absence of cells, formed the chief basis on which rested the humoral theory, which attributed the protection in such cases to the destructive action of the serum on the microbes. Flügge graphically illustrated the view of the humoralists by likening the phagocytes to the trenches made ready behind the fighting line to receive the conquered dead.

It was found, however, that cases of protection resulting from the use of immune serum occurred where no such bacteriolytic action could be demonstrated; the plague bacillus and the streptococcus may be mentioned as examples. Admitting that the phagocyte plays a part in the protection against these infections, the question must still be considered whether the immune serum has acted on the injected microbes or on the phagocytes. Metchnikoff maintains that the serum stimulates the leucocyte to its activity, whereas many workers, who are quite prepared to admit the important part which the phagocyte plays in the process, hold that the immune serum acts chiefly on the micro-organism.

Metchnikoff's view, however, is not opposed to the idea of the immune substance, or "substance sensibilisatrice," becoming fixed on to the cocci. He admits that this occurs, and that the micro-organisms thus sensitised, though they maintain their vitality and virulence, become more readily the prey of the leucocyte, whose activity is increased by the stimulating action of the "substance sensibilisatrice." In the animal body, under normal conditions, bacteriolysis of the microbe occurs within the phagocyte (Bordet and Levaditi). In experiments *in vitro*, or in the animal body where phagolysis has occurred, free cytase or complement being present, bacteriolysis may occur both outside and inside the phagocyte.

It may be of use here to make a brief reference to a few of the investigations carried out by the followers of Metchnikoff with reference to the influence exerted by the serum, on the one hand on the phagocytes, on the other hand on the microbes. The papers selected to illustrate the subject are by Bordet, Savtschenko, and Levaditi, and their views are referred to only in so far as they touch on these points.

Bordet (1895 and 1897) holds that the specific serum contains a thermostable substance, "sensibilisatrice," which acts on the micro-organisms and prepares them for the thermolabile alexin, or proteolytic ferment, which acts as the solvent. He compares the action of the immune serum to that of a mordant. In certain cases, however, such as in streptococcus infection, the bactericidal action is slight, and in such cases he attributes to the immune serum a stimulating action on the leucocytes. The leucocytes and other cells can perceive the presence of a preventive serum, and under its stimulus they are capable of reacting by movement. They manifest towards the immune serum a pronounced positive chemiotaxis. The activity of the leucocytes in the presence of such serum can be observed *in vitro*.

The bactericidal substance is not uniformly distributed through the plasma, but during life is confined within the leucocytes.

Savtschenko and Melkich (1901), from their study of the processes observed

in recurrent fever, come to the conclusion that the immune substance, or "fixateur," acts as an intermediary body between the micro-organism and the leucocyte, transforming the negative chemiotaxis of the latter into a positive chemiotaxis. They state that the "fixateur" may act in two ways:—

(1) The leucocytes may absorb the "fixateur," and acquire the chemical affinity necessary to enable phagocytosis to occur.

(2) The "fixateur," which is present in a free state in the plasma, becomes fixed on to the spirilla, to which it communicates the chemical affinity for the protoplasm of the leucocytes, and phagocytosis results.

The latter hypothesis is not invalidated by the fact that no Pfeiffer's phenomenon (of bacteriolysis) can be obtained by supplying alexin to the spirilla, since a much smaller quantity of "fixateur" may be necessary for phagocytosis than for bacteriolysis.

Savtschenko (1902), in a later work, comes to similar conclusions, based chiefly on experiments on the phagocytosis of red blood-corpuscles. The immune substance, or "fixateur," can fix itself on the microbe, or on the leucocyte, and has an affinity for the cytase contained in the leucocyte. It is probable, he thinks, that it acts as a stimuline for the phagocyte. He holds that it acts as an intermediary body between the leucocyte and the microbe, and merits fully Ehrlich's designation of "Zwischenkörper" (intermediary body).

Levaditi (1901) showed, by experiments *in vitro*, that cholera vibrios were ingested by the polynuclear leucocytes of the peritoneal exudate of a normal guinea-pig, and that the intracellular vibrios were converted into granules (intracellular, Pfeiffer's phenomenon), whereas the extracellular organisms remained unaltered. From a series of experiments, he came to the conclusion that this result was due to the presence of "substance sensibilisatrice," in sufficient quantity to enable phagocytosis to occur, the complement for the intracellular change of the vibrios into a globular form being, he believed, supplied from the leucocyte itself.

On the other hand, the extracellular solution of the microbes did not take place owing to a lack of complement. The leucocytic origin of the complement will not be approached in the present paper.

Levaditi also showed that the vibrios on to which the "substance sensibilisatrice" had been fixed when introduced into the circulation of a normal animal were rapidly phagocyted, just as they are in the case of an actively immunised animal. The extraphagocytic conversion of the vibrios into the granular form does not take place if sufficient precautions are observed to avoid injury to the leucocytes.

Denys and Leclef (1895) and Denys (1897) showed that the serum of rabbits immunised against streptococcus had a bactericidal action on the streptococcus, but that the serum of the horse had no such action, though it possessed protective properties. The immune substance in the one case acts as intermediary body between the cocci and the alexin; in the other case between the cocci and the leucocytes.

Denys (1897) made comparative tests of the phagocytic action of different sera *in vitro*. Measured quantities of streptococci were introduced into tubes containing leucocytes, and to certain of these were added immune serum, to others normal serum. By plating loopfuls taken from these tubes, and counting the colonies at various periods, he was able to demonstrate a marked diminution in the tubes containing the immune serum, whereas in the tubes containing the normal serum, an increase was observed.

He found that the leucocyte of the immunised animal was no more active as a phagocyte than the leucocyte of the normal animal. The difference in the two cases was entirely due to a property of the serum.

The conclusion arrived at was that the immunity of the rabbit against the streptococcus was due to a modification of the serum, which rendered phagocytosis possible. The immunity in this case is a humoral property acting by the intervention of the phagocytes.

Mennes (1897), using the same method of experimentation with the pneumococcus, obtained similar results to those obtained with the streptococcus by Denys, and concluded that the immunity in this case was due to a modification, not of the leucocyte, but of the serum. The serum had not acquired any bacteriolytic property, but had itself undergone a change, which resulted in the micro-organisms being taken up and destroyed by the leucocytes.

The results of Denys and also of Mennes are not altogether above the criticism made by Metchnikoff, viz., that the occurrence of a certain amount of agglutination would appear to give a diminution in the number of colonies.

Douglas and Wright (1903), adopting and modifying the method suggested by Leishman, have arrived at a very beautiful technique for the study of phagocytosis, and they have published a series of papers on the subject. The details of the method are too elaborate for reproduction here, but the essential point consists in enumerating the bacteria ingested in a number of polymorphonuclear leucocytes, and, by division, obtaining an average, which is taken as the measure of the phagocytic power of the blood. They find that there is present in the normal blood serum a substance which prepares the bacilli, so that they are capable of ingestion by the phagocytes. They call

this effect an "opsonic" effect ("Opsono," "I cater for, I prepare victuals for"), and they use the term "opsonin" to designate the element in the blood-fluids which produces the effect. They find that the "opsonin" is a thermolabile body, *i.e.*, is destroyed by heating to 60° C. for 10 minutes. The leucocyte is an indifferent factor in the matter.

As a result of inoculation with bacterial vaccines, the amount of the opsonin present in the blood may be increased.

Bulloch and Atkin (1905) have confirmed and extended these results of Wright and Douglas. They find that the "opsonin" disappears from serum when the latter is mixed with bacteria at 37° C. or 0° C. The action of heat is to destroy the "opsonin," and not merely to convert it into a "non-opsonisable" modification. It is a simple body, and is not identical with any of the antibodies hitherto discovered in the serum. Leishman, at a discussion on the subject at the Pathological Society, London, supported the "stimuline" view of the action of serum.

Neufeld and Rimpau (1904) find that the immune sera, in the case of the streptococcus and pneumococcus do not stimulate the leucocyte in Metchnikoff's sense, but act on and change the micro-organisms, so that they are secondarily taken up by the phagocytes. They find that the substance which produces this effect is thermostable. A polemic has been waged with Wright as to the identity of this body with the "opsonin" (*cf.* Savtschenko and Markl).*

Hektoen and Ruediger (1905) employed the method of Wright and Douglas, and confirm most of their results. They conclude that the "opsonin" has a complex constitution, there being present a haptophorous group, which fixes on the microbe, and an opsinophorous group, which produces a physical or chemical change in the microbe.

Introduction to Experiments.

The present investigation was undertaken with the view of studying certain questions as to the relation between the phagocytic immunity which occurs in normal animals and in those which have been actively immunised. The serum of a number of animals, chiefly horses, which have been immunised against various microbes, was examined and compared with the serum of normal animals of the same species. At first the method employed was Wright and Douglas', to which reference has already been made.

To save repetition, it may be stated that in almost every case equal volumes of washed leucocytes, of the serum and of the bacterial emulsion

* The writer had observed the thermostability of the substance in Staphylococcus immune serum and in normal serum before hearing of the work of Neufeld and Rimpau. Where the term "the substance" is employed in this paper, it is used as an abbreviation for "the substance which prepares the micro-organisms for phagocytosis."

were employed. The number of ingested cocci were enumerated in 22 polymorphonuclear leucocytes, and the average taken as the phagocytic index.

Estimations were made of the phagocytosis in the case of animals immunised against *Staphylococcus pyogenes aureus*,* *Streptococcus pyogenes*, *B. typhosus*, and *B. dysenteriæ*.

These experiments led to a closer study of the nature of the substance in serum which assists in the process of phagocytosis; and to some extent this enquiry resolved itself into a study of the relations which the immune substance, amboceptor, substance sensibilisatrice, or fixateur, bears to the opsonin of Wright and Douglas. For this investigation the *Staphylococcus pyogenes aureus* was chiefly employed owing to the convenience with which emulsions, counts, etc., could be made. Two races of the Staphylococcus were used, one highly virulent, recently isolated from the human body, the other an old laboratory culture. No marked differences were observed in the counts obtained from these.

Where the method of Wright and Douglas has been rigidly employed, the results I have obtained have been almost entirely in agreement with theirs. I should not feel disposed, however, to place quite the same reliance as they do on the numerical accuracy of the results which can be derived from the method. Where the leucocytes are very full—*i.e.*, where the counts are high—it is impossible to differentiate results by the method of enumeration. In this paper differences obtained by all refinements of enumeration have been neglected. In all cases an excess of cocci was present. The majority of the counts have been made by two or three observers who did not know the objects in view, so that personal bias was eliminated. Without entering into details, it may briefly be stated that, when using their method, my experiments tend to confirm theirs in that there is in normal serum a substance which prepares the microbe for the leucocyte; that this substance, or "opsonin," as tested by Wright's method, appears to be destroyed at a temperature of 60° C. for 15 minutes, and that the leucocyte is, in a certain sense, an indifferent factor. By the employment of other methods, results in oppositition to Wright and Douglas' have been obtained.

When, however, the question of the thermolability of the "opsonin" was investigated in another manner, the results obtained were opposed to the view that the substance was destroyed at the temperatures named by Wright. Indeed, it was found that the heated immune sera, even on testing by Wright's method, gave high phagocytic indices. These results suggested a re-investigation of the effects of temperature by other methods.

* Referred to hereinafter as "Staphylococcus."

One method employed was that of adding a measured quantity of a microbial emulsion to a certain volume of serum which had been heated to 60° for 20 minutes, allowing this to stand for varying periods at 37° C., then centrifugalising and ascertaining whether the centrifugalised cocci were capable of being taken up by the leucocytes.

Experiment.—0·5 c.c. of an emulsion of Staphylococcus (= ½ agar tube) was added to 5 c.c., of a normal horse serum, which had been heated to 60° C. for 20 minutes. The cocci, separated by centrifugalisation and made into a suitable emulsion with normal salt solution, were compared with normal fresh cocci in Wright's tubes.

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Emulsion of fresh cocci	1	
+Heated normal horse serum	1	4
+Normal horse leucocytes.....	1	
Emulsion of prepared cocci.....	1	
+Normal salt solution	1	60
+Normal horse leucocytes.....	1	

Experiment.—0·1 c.c. of an emulsion of Staphylococcus (= 1/10 of an agar tube) was mixed with 1 c.c. of normal human serum, which had been heated to 60° C. for 20 minutes. This mixture was placed for 15 minutes at 37° C., and then the cocci were centrifugalised from the mixture and made into a suitable emulsion with normal salt solution.

These prepared cocci and fresh cocci were used for a comparative test in capillary tubes in the ordinary manner.

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Fresh cocci.....	1	
+Heated normal serum	1	0
+Leucocytes (human).....	1	
Prepared cocci	1	
+Normal salt solution	1	60
+Leucocytes (human).....	1	

A large number of similar experiments which need not be detailed here were done with concordant results; in several cases a smaller proportion of serum to bacterial emulsion was used.

Such results were obtained in the cases of normal human serum (four samples), horse serum, goat serum, rabbit serum, guinea-pig, and rat serum. Normal horse serum which had been kept for four years still retained this property.

These experiments prove that though the results obtained by using Wright's method seem to demonstrate that the substance capable of preparing the

microbes for phagocytosis is destroyed by heating for 20 minutes in 60° C., only a fractional destruction of the substance occurs. The loss in the case of sera, such as normal sera, which contain only a comparatively small quantity of the substance, is so great that a method where very small quantities are used makes the demonstration and estimation of the substance impossible.

On the other hand, where a large amount of the substance is present, as in certain immune sera, the heating to 60° C. for 20 minutes, or for even much longer periods, leaves enough of the substance undestroyed to give results by Wright's method. Indeed, the counts obtained may be higher than with fresh normal serum of the same species.

One or two experiments may be quoted as examples of a large number done which gave consistent results :—

Experiment.—

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Fresh normal rabbit's serum	1	
+Suspension of Staphylococcus.....	1	9
+Leucocytes of horse	1	
Heated normal rabbit's serum	1	
+Suspension of Staphylococcus	1	0·2
+Leucocytes of horse	1	
Immune rabbit's serum (heated to 60° C. for 20 minutes)	1	
+Suspension of Staphylococcus	1	20
+Leucocytes of horse	1	

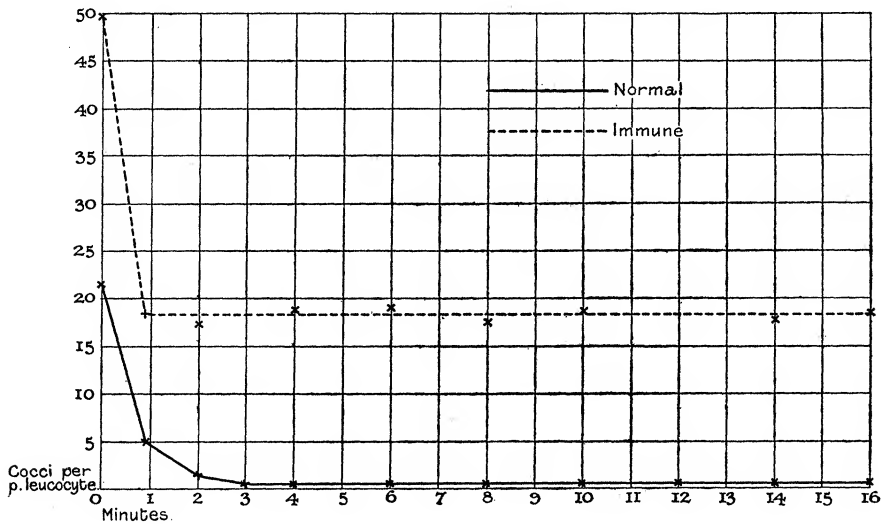
The serum of the normal horse, even when heated to 60° C. for 20 minutes, gave in certain cases, by Wright's method, fairly high counts. It contained a larger quantity of the substance than heated normal rabbit's serum.

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Fresh normal horse serum	1	
+Suspension of Staphylococcus	1	30
+Leucocytes of horse	1	
Fresh normal horse serum (heated to 60° C. for 20 minutes).....	1	
+Suspension of Staphylococcus	1	8
+Leucocytes of horse	1	
Immune heated serum of horse	1	
+Suspension of Staphylococcus	1	60+
+Leucocytes of horse	1	

These experiments show that in an immune Staphylococcic serum enough of the substance remains undestroyed by heating to be demonstrable by the use of Wright's method.

When fresh serum, either normal or immune, is heated to 60° C. for various periods, one finds that, as estimated by Wright and Douglas' method, there is a great fall in the first two minutes, and after that the curves run almost parallel to the base line. In the case of normal serum the fall is so great that the curve may reach the base line.

The accompanying chart gives a graphic representation of what happened



on heating a normal rabbit's serum and an immune Staphylococcus rabbit's serum for different periods up to 16 minutes. The number 50 applied to the original strength of the immune serum is only approximate, as in such high counts accuracy is impossible. It will be seen that after one minute's heating the immune serum fell to about 18 cocci per leucocyte, and, allowing for experimental errors (the average of a large number of counts was taken), it then runs parallel to the base line.

The normal serum fell from 20 to 5 at the end of the first minute, to 1 at the end of the second minute, and then ran almost parallel to the base line, the average phagocytic index of a number of counts being about 0·5.

The results after 30 minutes showed that no great change had occurred.

One must remember that Wright and Douglas' method probably demonstrates the presence of the "opsonin" over only a very short range. The brief time during which the substance is allowed to act on the cocci probably admits of only fairly high concentrations being indicated. If one compares

what occurs in the case of agglutination, only the very strongest agglutinating sera would be regarded as giving positive results if 15 minutes were put as the limit of time for the serum to act on the microbes.

As has been shown by the experiments quoted, the fall in the curve does not indicate complete destruction of the substance, as was stated by Bulloch and Atkin, but merely indicates that the substance has reached a concentration below that demonstrable by Wright and Douglas' method.

This view is emphasised by the experiment detailed in the following section.

Effect of Continued Heating on the Substance in Serum.

Two series were prepared with normal horse serum heated at 60° C. for different periods. In each tube 1 c.c. of the heated serum was placed, and to the tubes of the one series 0·08 c.c., to those of the other 0·05 c.c., of a coccal emulsion was added. The mixtures in each case were put for 15 minutes at 37°, then centrifugalised, and the centrifugalised cocci tested in the usual way. In most cases the leucocytes had to be noted simply as full, but where an obvious fall in the number of ingested cocci was observed, an enumeration was made.

Time for which serum was heated at 60° C.	Tubes to which 0·08 c.c. coccal emulsion added. No. of cocci per leucocyte.	Tubes to which 0·05 c.c. coccal emulsion added. No. of cocci per leucocyte.
20 minutes	Full	Full
40 ,,	Full	Full
60 ,,	Full	Full
2 hours	36	Full
3 ,,	30	44
4 ,,	30	21

This experiment proves that normal horse serum after being heated for four hours at 60° C. still contains enough of the substance to prepare a large number of the cocci for phagocytosis. With the quantities of coccal emulsion employed in this experiment a fall was observable with the larger quantity only after two hours' and with the smaller quantity after three hours' heating.

On the Influence of Temperature on the Rate of Combination between the Substance and the Cocci.

0·1 c.c. of an emulsion of Staphylococcus was added to 2 c.c. of normal human serum, which had been heated to 60° C. for 25 minutes. The emulsion was at once divided into two parts, and one part placed at 37° C., and the other at about 6—8° C., in the ice-chest. At the end of half an hour they were centrifugalised, the centrifuge buckets having been cooled down. The centrifugalised cocci in the two cases were then tested.

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Coccal emulsion which had been kept at 37° C.	1	22
+Human leucocytes	1	
Coccal emulsion which had been kept at 6—8° C.	1	2·2
+Human leucocytes	1	

This experiment seems to show that the substance acts on or becomes attached to the cocci much more slowly at low than at high temperatures.

Microbes which have been placed in Contact with Immune Serum are capable of taking up an Excess of the Substance which can again Diffuse into the Surrounding Fluid.

0·5 c.c. of an emulsion of Staphylococcus was put in contact with 2 c.c. of a heated immune serum and left in contact at 37° C. for 1 hour. The cocci were then separated by centrifugalisation, washed and put into 1 c.c. of an immune serum, X, and left in contact for an hour at 37° C., then centrifugalised very carefully so that all the cocci were thrown down.

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Fresh serum "X"	1	37
+Staphylococcic emulsion	1	
+Washed horse leucocytes	1	
Serum, X, through which prepared cocci have been centrifugalised	1	48
+Staphylococcic emulsion	1	
+Washed horse leucocytes	1	

This points to a diffusion of the substance into the serum from the cocci.

Experiment.—Cocci which had been treated, as in the previous experiment, with immune serum, after being centrifugalised and rapidly washed with normal salt solution, were left in contact for 24 hours with normal salt solution, which was then tested for the presence of "opsonin."

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Washed leucocytes of horse	1	0·5
+Normal salt solution	1	
Washed leucocytes of horse	1	21
+Normal salt solution, from above prepared cocci	1	

A number of experiments were carried out with the view of ascertaining the relation of the substance in normal serum to the substance in immune serum.

Experiment to ascertain whether Cocci fully occupied by the Substance from Normal Serum are capable of absorbing the Substance from Immune Serum.

An emulsion of Staphylococcus, prepared in the usual manner, was added to normal horse serum which had been heated for 25 minutes to 60° C. The mixture was placed at 37° C. for 15 minutes, and the cocci were then centrifugalised till all were removed from the fluid. The supernatant fluid was then removed and the cocci rapidly washed with normal salt solution and again thrown down, great care being taken to avoid any loss of the cocci during the process.

To equal parts of a certain Staphylococcus immune serum from the horse, also heated to 60° C. for 20 minutes, there were added equal parts of the sensitised cocci and of fresh normal cocci. These mixtures were then centrifugalised, and the phagocytic indices of the supernatant fluids estimated and compared, the usual proportions of washed leucocytes and Staphylococcus emulsion being used.

	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Original heated immune serum	24
Original heated immune serum through which prepared cocci had been centrifugalised	13
Original heated immune serum through which normal cocci had been centrifugalised	4

These numbers are the mean of three counts each of 22 polymorphonuclear leucocytes.

A similar experiment carried out with a dilution of one in five of a similar serum of greater potency gave the following numbers :—

	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Phagocytic index of the dilution of original Staphylococcic immune serum of horse	30
Ditto through which prepared cocci had been passed	24·3
„ „ normal „ „	8

These two experiments seem to show that the cocci occupied by the substance from normal serum are incapable of taking up the substance from immune serum, whereas fresh cocci are capable of removing a large proportion of the substance.

The converse experiment was carried out. In this case the cocci were first prepared by contact with immune serum and then passed through a normal serum. For convenience of estimation by Wright's method, instead of a normal heated serum a normal fresh unheated serum was employed.

Equal measured quantities of fresh cocci and of cocci prepared as in the previous experiments by contact with heated immune serum of the horse were added to equal

volumes of normal horse serum, centrifugalised, and the supernatant fluid, which was then free from all organisms, tested for phagocytic power, normal washed horse leucocytes being used.

	Average number of cocci per leucocyte in 22 polymorphonuclear leucocytes.
Original serum	40
Serum through which prepared cocci had been passed	30
Serum through which fresh normal cocci had been passed	2·3

The repetition of this experiment with freshly prepared material gave the following numbers :—

	Average number of cocci per leucocyte in 22 polymorphonuclear leucocytes.
Freshly prepared serum	35+
Ditto through which the prepared cocci had been passed	27
Ditto through which fresh normal cocci had been passed	2

In carrying out these experiments care must be taken that excess of substance is not left adhering to the cocci, in which case it may diffuse out into the surrounding fluid. See previous experiments. To obviate this happening the prepared cocci must be rapidly washed in normal salt solution.

These experiments seem to show that the cocci occupied by the substance from immune serum are incapable of taking up much of the substance from normal serum, whereas fresh cocci are capable of removing a large proportion of the substance.

Tests of the Serum of Animals Immunised against the Streptococcus, Typhoid and Dysentery Bacilli.

Streptococcus.

The serum of three horses immunised against many races of streptococci was examined with the following result :—

	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Horse 1—		
Washed leucocytes of horse	1	
+Serum of immune horse	1	9
+Emulsion of streptococcus from agar	1	
Washed leucocytes of horse	1	
+Serum of normal horse	1	4
+Emulsion of streptococcus from agar	1	
Horse 2—		
Washed leucocytes of horse serum	1	
+Serum of immune horse	1	12
+Emulsion of streptococcus from agar	1	

Horse 2—*continued.*	Vol.	Cocci counted in 22 leucocytes. No. of ingested cocci per leucocyte.
Washed leucocytes of horse serum	1	
+Serum of normal horse	1	3
+Emulsion of streptococcus from agar	1	
Horse 3—		
Washed leucocytes of horse serum	1	
+Serum of Horse 3	1	23
+Emulsion of streptococcus from agar	1	
Washed leucocytes of horse serum	1	
+Serum of normal horse	1	4
+Emulsion of streptococcus from agar	1	

Typhoid Bacillus.

	Vol.	Bacilli contained in 22 polymorphonuclear leucocytes. No. of ingested bacilli per leucocyte.
Serum of normal horse heated to 60° C. for 20 minutes	1	
+Washed horse leucocytes	1	22
+Emulsion of typhoid bacillus	1	
Serum of immune horse heated to 60° C. for 20 minutes	1	
+Washed horse leucocytes	1	49
+Emulsion of typhoid bacillus	1	

Dysentery Bacillus (Shiga).

Serum of normal horse heated to 60° C. for 20 minutes	1	
+Washed horse leucocytes	1	1·5
+Emulsion of dysentery bacillus	1	
Serum of immune horse heated to 60° C. for 20 minutes	1	
+Washed horse leucocytes	1	25
+Emulsion of dysentery bacillus	1	

In the case of the typhoid and dysentery bacilli considerable difficulty was found in estimating the phagocytosis on account of the agglutination. The leucocytes, crammed full of bacilli, were found in some cases lying in groups close to bacillary clumps, in other cases it seemed as if the leucocytes when full of microbes tended themselves to become agglutinated.

It is possible that an agglutination of the bacilli towards the leucocytes may be a part of the process which enables the leucocyte by the movements of its own protoplasm to englobe the microbe. The extraordinary rapidity

with which a bacterial field is cleared of micro-organisms suggests such an occurrence.

An interesting observation was made in this connection, viz., that organisms after being stained with fuchsin, which has a strong agglutinating action, were capable of ingestion, whereas the same organisms killed by heat were refused by the leucocyte.

It was observed that the normal serum of the same horse heated to 60° C. for 20 minutes had a considerable "opsonising" action on the typhoid bacillus, whereas it had little effect on the dysentery bacillus. Twenty-two typhoid bacilli were ingested per leucocyte compared with 1·5 dysentery bacilli.

Summary and Remarks.

An immune staphylococcic serum obtained from the rabbit when heated to 60° C. still contains a substance capable of preparing the cocci for phagocytosis. An identical result was obtained in the case of an immune staphylococcic serum from the horse. Efforts were made to ascertain whether this substance was identical with the substance in normal serum giving rise to the same effect, *i.e.*, to the "opsonin" of Wright. It was found that when the cocci were added to a fairly large volume of normal serum which had been heated to 60° C. for 20 minutes, incubated for 15 minutes and centrifugalised, they were rapidly phagocyted.

It appeared, therefore, that the heating to 60° C. had produced only a fractional destruction of the opsonin, which in the case of normal serum was present in such small amount that it was no longer measurable by Wright and Douglas' method.

This view was confirmed by heating for various periods both normal and immune serum and comparing the fall resulting. The curves obtained in both cases were similar. The serum of various animals, the goat, rabbit, horse, guinea-pig, rat, and human were tested for the thermostability of the substance. In all it was found to be thermostable when tested in the way mentioned, but more of it appeared to be present in the serum of the horse than in the serum of, *e.g.*, man and rat, since in the case of the horse it was found to be present, after heating, in an amount which was still capable of being demonstrated by Wright's method. Horse serum heated for four hours to 60° C. still contained a large amount of the substance.

Experiments were carried out with the view of ascertaining the relation of the substance in normal serum to the substance in immune serum.

Cocci which had been prepared by contact with normal serum so that they were probably fully occupied by the substance were passed through heated

immune serum. On removal of the cocci by the centrifuge the supernatant fluid, when tested with fresh cocci, was found to have lost little, or none, of its original strength; whereas the same fluid through which fresh cocci had been centrifuged had lost practically all its power. The converse experiment gave a quite similar result. In this case, however, care must be taken to avoid the cocci being overloaded with immune serum, in which case they are capable of giving off some of their substance into the suspending fluid.

In this case also it is more convenient to use unheated normal serum, which enables one to easily estimate the opsonic power by Wright and Douglas' method. These two groups of experiments are strongly in favour of the substance in normal serum being identical with the substance in immune serum.

The relation which the "opsonin" of Wright and Douglas bears to the "immune substance," or "fixateur" in so far as the latter influences phagocytosis, as shown by Savtschenko among others, must be briefly discussed.

If the "opsonin" of normal serum were completely destroyed by heating to 60° C. for 15 minutes we should be compelled to assume that it was a separate and new body, and that the increase in the serum of the property of preparing the microbes for phagocytosis, which results from the injection of bacilli, was due to an entirely different body, since the substance resulting from such bacterial injections is markedly thermostable, even when tested by Wright and Douglas' own method.

The experiments which are recorded in this paper, however, show that the destruction by heating of the "opsonin," even of normal serum, is only fractional, and that its apparently complete disappearance is due to the method of observation employed, which demonstrates its presence over a very short range. According to the ordinary use of the word in such investigations the body is thermostable.

The fact that this specific substance is present in small amount in normal serum is in accord with the numerous observations of the occurrence of immune substance in normal sera. One need only refer to the normal antitoxin (*e.g.*, of diphtheria), anti-ferments, etc., and to the fact that the bacteriolytic and hæmolytic actions of normal serum are due to the presence in the serum of an immune substance plus a complement, as has been firmly established by the work of Pfeiffer, Bordet, Moxter, Ehrlich and Morgenroth, and others. In giving the name of "opsonin" to the substance which becomes attached to the micro-organisms and prepares them for phagocytosis, Douglas and Wright have, therefore, named a property of serum which had already been recognised by a number of different workers.

Whether free complement may take part in the preparation of the microbe is difficult to determine. From the experiments detailed in this paper it is

certain that it is not a necessary participant in this action. At the same time it is not improbable that the immune body when aided by complement may act more powerfully, and that the sudden fall in the "opsonic" power of both normal and immune serum on heating is due to the destruction of the complement. I may revert to this subject on another occasion.

Metchnikoff's statement in regard to natural immunity, that the leucocytes undertake the struggle against the microbes and free the organisms from them without the need of previous help on the part of the humors, is apparently largely based on Bordet's and Gengou's results obtained by their method of testing for the presence of "fixateur." The results obtained by the use of that method are not above criticism. That the amount of fixateur present in normal serum compared with immune serum is small is true, but, as suggested by Savtschenko, the amount necessary to prepare the organism for phagocytosis may be small compared with that necessary for bacteriolysis. When microbes, *e.g.*, streptococci, injected into the peritoneal cavity, come in contact with the phagocytes, at first they are englobed by these, but soon some are observed to be free and to multiply rapidly, apparently having the power of repelling leucocytes. Bordet and Savtschenko interpret this to mean that the cocci have acquired during this brief period a new property which gives rise to a negative chemiotaxis of the leucocytes.

In the light of my experiments another view may be taken, viz., that the first organisms injected are phagocyted, because they have been sensitised by the immune substance present in the normal serum. This being small in amount is soon exhausted, and the few organisms which may have escaped its action are able to multiply, and either are indifferent to the leucocytes or exercise their repelling influence on them in the absence of the naturally present immune substance. In such a case the indifference displayed by the phagocytes to the cocci, or it may be the repulsive force of the cocci, is not a newly-acquired property, but is inherent in the cocci, and is only overcome by the presence of the immune substance which acts as intermediary between the micro-organism and the leucocytes.

Conclusions.

1. That, as has been shown by a number of workers, *e.g.*, Denys, Metchnikoff, Savtschenko, Levaditi and others, there is produced in the blood serum of animals actively immunised by bacterial injections a specific immune substance which has among its properties that of preparing the microbe for phagocytosis.

2. That this immune substance is thermostable, resisting a temperature of 60° C. for several hours.

3. That in normal serum there is present a substance having a similar action and which also resists a temperature of 60° C. for hours, and may persist in the serum of the horse for years.

4. That the experiments recorded in this paper tend to confirm the idea that the substances are identical, *i.e.*, that in normal serum there is present a small amount of the immune substance having the property of preparing the microbes for phagocytosis.

5. That cocci fully occupied by the substance from heated immune serum when passed through fresh normal serum do not remove the substance from normal serum, whereas fresh cocci remove a large part of it.

6. That the converse of the above is also true, viz., that cocci fully occupied by the substance from normal serum do not remove the substance from immune serum, whereas fresh cocci do.

7. That the thermostable substance in normal serum is no doubt identical with the "fixateur" or "substance sensibilisatrice" of the French school and with Wright and Douglas' "opsonin."

Seeing that the terms "fixateur" and "substance sensibilisatrice" which have been employed by Metchnikoff's school to include the property of preparing the microbes for phagocytosis are used to designate a number of other properties of immune serum, it may be convenient to adopt Wright and Douglas' term of "opsonin" for the particular property in question. The only danger attached to such a course is that one might be led to regard the "opsonin" as actually a different substance and not merely a property of immune serum.

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The Phagocytosis of Red Blood-Cells.

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It has been shown by Savtschenko,* that when an animal has received injections of red blood-cells, its serum (inactivated by heating) causes the appearance of phagocytosis *in vitro* when leucocytes and red blood-cells of the kind used for injection are added, and the whole maintained at the temperature of the body. This action is attributed by Savtschenko to the action on the red blood-cells of amboceptor (immunisine, fixateur) contained in the serum employed.

In order to obtain further information respecting the factors which determine phagocytosis *in vitro* of red blood-cells, a comparative examination of the action of sera of animals nearly related to, and widely separated from, those supplying the erythrocytes used for injection was undertaken. This investigation confirmed the above observation, that leucocytes placed in the inactivated serum of injected animals ingested red blood-cells of the kind used for injection. In the course of this investigation, however, it was found that phagocytosis could be brought about by the serum of the injected animals when the serum was free from amboceptor, for the red blood-cells injected, as the following experiment shows:—

Experiment 1.—Into the abdominal cavity of a dove, the red blood-cells

* "Du Rôle des Immunesines (Fixateurs) dans la Phagocytose," 'Annales de l'Institut Pasteur,' 1902, vol. 16, p. 106.